FORM PTO-1390 (REV. 1-98) U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: BO 41480

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/486073

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL97/00474	21 August 1997	--

TITLE OF INVENTION
NOVEL MUTANTS OF GRAM NEGATIVE MUCOSAL BACTERIA AND APPLICATION THEREOF IN VACCINES

APPLICANT(S) FOR DO/EO/US
Peter Andre VAN DER LEY and Liana Juliana Josephine M. STEEGHS

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau. (see PCT/IB/308)
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

International Preliminary Examination Report.

Search Report.

Form PCT/IB/308.

Inventor information sheet.

514 Rec'd PCT/PTO 22 FEB 2000

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/486073	PCT/NL97/00474	BO 41480

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

		CALCULATIONS PTO USE ONLY	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$ 970.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$840.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$760.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$670.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 840	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$ 130	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18 - 20 =	0	x $18.00	$ 0	
Independent claims	2 - 3 =	0	x $78.00	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 970	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).			+	$	
SUBTOTAL =				$ 970	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 970	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	$	
TOTAL FEES ENCLOSED =				$ 970	
				Amount to be refunded:	$
				charged:	$

a. [X] A check in the amount of $ 970 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required by 37 CFR 1.16 and 1.17, or credit any overpayment to Deposit Account No. 25-0120. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

Customer No. 000466

February 22, 2000

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PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Peter Andre VAN DER LEY et al.

Serial No. (unknown)

Filed herewith

NOVEL MUTANTS OF GRAM NEGATIVE MUCOSAL BACTERIA AND APPLICATION THEREOF IN VACCINES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to calculation of the filing fee, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please substitute pages 1 and 2 of the specification as originally filed with pages 1 and 2 as filed in the Article 34 amendment of October 15, 1999. The pages are marked "AMENDED SHEET" and are attached hereto.

IN THE CLAIMS:

Please substitute Claims 1-20 as originally filed with Claims 1-18 as filed in the Article 34 amendment of October 15, 1999, and which are attached hereto and marked "AMENDED SHEET". Following the insertion of Claims 1-18, please amend these claims as follows:

Claim 3, line 1, cancel "or 2".

Claim 4, line 1, change "any one of claims 1-3" to --claim 1--.

Claim 5, line 1, change "any one of claims 1-3" to --claim 1--.

Claim 6, line 1, cancel "or 2".

Claim 7, line 1, change "any on of the preceding claims" to --claim 1--.

Claim 8, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 9, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 10, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 11, line 2, change "any one of claims 1-10" to --claim 1--.

Claim 12, line 2, change "any one of claims 1-10" to --claim 1--.

Claim 13, line 2, change "any one of claims 1-10" to --claim 1--.

Claim 14, line 1, change "f claims 11-13" to --of claim 11--.

Claim 15, line 1, change "any one of claims 11-14" to --claim 11--.

Claim 16, line 2, change "any one of claims 1-10" to --claim 1--.

Article 34 Amdt

Novel mutants of gram negative mucosal bacteria and application thereof in vaccines.

Summary of the invention

We found that contrary to previous findings with *E. coli* it is possible to inactivate the early stage of lipid A synthesis of mucosal gram negative bacteria without compromising cell viability. In particular the *lpxA* gene in *Neisseria meningitidis* was mutated without compromising cell viability. The resulting *lpxA* knockout mutants were found to be completely LPS-deficient. The major outer membrane proteins (OMPs) were detected in normal amounts. Also, an outer membrane could be discerned in electron micrographs of ultrathin sections. To our knowledge, this was the first instance of a viable Gram-negative bacterial mutant completely lacking in LPS.

The finding provides important implications for our understanding of structure and biogenesis of the outer membrane. On a practical level, the availability of LPS-deficient mutants of pathogenic mucosal bacteria such as *N. meningitidis* opens up new avenues to vaccine development. It enables easy isolation of endotoxin-free purified proteins, outer membranes or even whole-cell preparations for use in immunisation.

Background information

Lipopolysaccharide (LPS) constitutes the outer monolayer of the outer membrane of Gram-negative bacteria. As such it forms an important component of the outer membrane and has been considered relevant for vaccine purposes (Verheul et al, 1993). The membrane-anchoring lipid A part is responsible for the well-known endotoxin activity of the molecule (Zähringer et al., 1994).

Such endotoxin activity is undesirable in vaccines. Currently some preparations to be used in vaccines are subjected to rigorous, time consuming and costly purification procedures in order to remove this endotoxin activity prior to their being suitable for use as a vaccine. This allows higher doses due to reduced toxicity. However, drastic purification methods can easily lead to denaturation of protein antigens which need to retain their native conformation in order to induce an appropriate immune response. To date Group A and C polysaccharide vaccines are available which have been rendered substantially free of lipo-polysaccharide by means of purification. To date however no whole cell vaccines substantially free of LPS nor OMP vaccines substantially free of LPS have been produced.

The isolation of the *N. meningitidis lpxA* gene involved in lipid A biosynthesis has recently been reported (Steeghs et al., 1997). The deduced amino acid sequence of the LpxA protein showed homology to the *E.coli* acyltransferase responsible for adding the *O*-linked 3-OH myristoyl chain to UDP-N-acetylglucosamine, which is the first committed step in the lipid A biosynthetic pathway (Anderson and Raetz, 1987; Coleman and Raetz, 1988). Based on this homology and a comparison of the *E.coli* and *N.meningitidis* lipid A structures it is expected that the meningococcal *lpxA* gene encodes an acyltransferase with 3-OH lauroyl specificity (Kulshin et al., 1992). The basis of the different fatty acid specificity might conceivably be located in the characteristic hexapeptide repeat motif of these acyltransferases which has been determined to play a crucial role in the folding of the *E.coli* protein (Vuorio et al., 1994; Raetz and Roderick, 1995). In an attempt to verify this hypothesis we constructed a hybrid *lpxA* gene in which the meningococcal N-terminal ß-helix domain containing the hexapeptide repeat motif was replaced by the corresponding part of *E.coli lpxA*, followed by transformation and allelic replacement of this construct to *N. meningitidis* H44/76. The experimental data for this are provided in the examples (in particular example 1).

The results demonstrated that strain H44/76[pHBK30] is a viable LPS-deficient mutant. The most likely explanation for this surprising discovery seemed to be that the hybrid *lpxA* gene had become inactive, either because of disrupted transcription/translation in our construct, or else because the hybrid protein as expressed had lost its enzymatic activity. To discern this, we constructed an *lpxA* knockout mutant. The results demonstrated once more that blocking of the lipid A biosynthesis pathway in *N. meningitidis* strain H44/76 leads to viable LPS-deficient mutants.

This is the first report of a viable Gram-negative bacterial mutant completely deficient in LPS. It has the following implications:

(1) Surprisingly (in view of the above mentioned view of the essential nature of lipidA biosynthesis for cell viability), it is possible for some gram negative bacteria to make an outer membrane without any LPS yet remain viable. Although our results do not exclude an involvement of LPS in the OMP forming process, they do demonstrate that it obviously cannot be essential. It should be very interesting to study the structure of the outer membrane in the *lpxA* mutant in more detail.

(2) In *E.coli*, all mutations affecting the early steps of lipid

A biosynthesis that have been described are lethal when expressed. The fact that this is not the case in Meningococci opened up the question which organism is typical in this respect, and what causes this difference. Conceivably, it is related to a different LPS-OMP interaction, which is also suggested by the observation that whereas deep-rough LPS mutants of *E.coli* and *Salmonella typhimurium* show a reduced expression of the major OMPs (Koplow and Goldfine, 1974; Ames et al., 1974), a comparable heptose-deficient *rfaC* mutant of *N.meningitidis* was found to have normal expression of the class 1 and 3 porins (Hamstra and van der Ley, unpublished).

We postulate that mucosal gram negative bacteria can in an analogous manner be mutated thereby becoming free of endotoxic LPS. Subsequently enabling development of LPS free whole cell or acellular vacines such as OMP vaccines. The basis for this postulation is found in the knowledge available to the skilled person concerning the Lipid a biosynthesis in mucosal gram negative bacteria. Figure 6 e.g. as derived from Raetz 1990 provides a diagram of the early steps in lipid A biosynthesis. It reveals the requirement of *lpxA* and *lpxB* as enzymes required in the early biosynthesis. The enzyme *lpxD* is also known to be involved (Steeghs et al 1997). Knowledge of the nucleic acid sequences encoding these genes is available to the skilled person (Steeghs et al 1997). Subsequently mutating one or more of the genes encoding the enzymes involved in the early stages of LipidA biosynthesis is possible. Figure 6 shows the early stages; preferably the mutation will arise such that no stage leading past the *lpxB* stage is reached as these products already closely resemble Lipid A structure. Preferably the mutation will arise as early as possible in the biosynthesis pathway. In most cases the genes encoding *lpxA*, *lpxB* and *lpxD* are clustered. Steeghs et al provides references disclosing such details for *Escherichia coli*, *Salmonella typhimurium*, *Yersinia entereocolitica*, *Haemophilus influenzae* and *Rickettsia rickettsii*. Knowledge of the sequences of these microorganisms is thus available to the person skilled in the art and homologous sequences in other organisms can be found. Both *lpxA* and *lpxD* contain a characteristic hexapeptide repeat structure $[(I,V,L)GXXXX]_n$. The *lpxB* gene is generally cotranscribed with *lpxA* and as such can also be readily found. The cluster also comprises the *fabZ* gene which can also be used to ascertain the location of the gene cluster involved in Lipid A biosynthesis (Steeghs et al 1997). Steeghs et al provide the genbank number under which the *N.meningitidis* sequence data concerning the Lipid

to enhance the immunogenic activity thereof. A number of adjuvants commonly used in vaccines are known. Any of these can be suitably applied. Any dosage form and additional components commonly used for vaccines, in particular meningococcal vaccines is suitable for the subject invention.

Particularly suited target microorganisms are diplococci and *Bordetella pertussis*. The diplococci comprise meningococci and gonococci. Examples of each category are *N.meningitidis and N. gonorrhoeae*. Numerous other organisms falling within this category are known from Bergeys Handbook of Determinative Bacteriology. These diplococci are structurally closely related and show the same gene structure. Both are interesting microorganisms from a vaccination view point as are a number of other microorganisms such as *Haemophilus influenzae* and *Moraxella catarrhalis*.

Clearly, the construction of *lpxA* knockout mutants can be attempted in other bacterial species known to have LipidA in their lipopolysaccharide.

(3) The availability of LPS-deficient mutants will allow new approaches to vaccine development against *N.meningitidis* and the closely related pathogen *N.gonorrhoeae*, as well as any other bacteria as mentioned above for which such mutants can be made and isolated. First, it will become much easier to purify OMPs or other cell surface components without contaminating endotoxin. Secondly the role of LPS in meningococcal outer membrane vesicle vaccines, e.g. as adjuvant or in stabilising OMP conformation (Verheul et al., 1993; Nakano and Matsuura, 1995; Poolman, 1995), can now be unequivocally determined and possibly taken over by a less toxic compound. Thirdly the use of inactivated whole cell vaccines can be investigated using endotoxin-free mutants according to the invention such as the *lpxA* mutants. Finally, the possibility to use LPS-deficient strains as live attenuated vaccines now arises.

The exact nature of the invention will be further elucidated with the following examples.

Example 1: Construction of an inactive *lpxA* gene in *N.meningitidis*

In two separate PCR reactions the *E.coli* and *N.meningitidis* part of the hybrid gene were amplified with the Epr1/Epr2 and Npr1/Npr2 primer, respectively (fig.1). The inside primers Epr2 and Npr1 were designed so that the ends of the products contain complementary

sequences. These products were mixed, denatured and reannaeled in a second PCR in which the fused construct was amplified by the outside primers Epr1 and Npr2, having an *MluI* and *SpeI* site respectively (fig.1). The resulting PCR product was cloned and its sequence verified.

To test the activity of the hybrid *lpxA*, this gene was used to replace the original *lpxA* in the meningococcal chromosome (fig.2). For this purpose the 1.0 kb *MluI/SpeI* fragment carrying the wildtype *lpxA* gene in plasmid pLA19 (a pUC18 derivative with a 1.9 kb *lpxD-fabZ-lpxA* insert) was replaced by the similarly digested hybrid *lpxA* gene. Subsequently, a kanamycin-resistance cassette was ligated into the *MluI* site located directly upstream of *lpxA*, resulting in the plasmid pHBK30.

Transformation of *N.meningitidis* H44/76 with linearized pHBK30 yielded kanamycin-resistant colonies after 24 hours of incubation. These mutants died when transferred to fresh GC plates with kanamycin (100 µg/ml).

By reducing the kanamycin concentration and screening of the resulting colonies by PCR amplification of *lpxA* hybrid-specific fragments we finally succeeded in the isolation of viable *kanR*$^{\pm}$ H44/76[pHBK30] transformants in which the chromosomal *lpxA* gene had been replaced by the hybrid construct as shown in fig.2.

LPS of the H44/76[pHBK30] mutant and the wildtype strain was compared by Tricine-SDS-PAGE followed by a silver stain for carbohydrates (fig.3). Surprisingly, no LPS could be detected in the hybrid derivative by this method, even when higher amounts of cell lysates were loaded on the gel.

To get more insight into the structure of the outer membrane of H44/76[pHBK30] a panel of LPS and OMP specific mAbs was tested in a whole cell ELISA (Table 1). The mutant strain did not bind any of the LPS-specific mAbs, whereas the OMP-specific mAbs showed similar binding patterns for mutant and wildtype. This apparent OMP similarity was confirmed when OMCs of H44/76[pHBK30] and H44/76 were isolated and analysed by SDS-PAGE (fig.3). Both strains show equal amounts of the class 1, 3 and 4 OMP; in contrast to the wildtype, the mutant apparently also expresses a class 5 OMP.

Since LPS of H44/76[pHBK30] could not be detected with any of the methods described above, it became questionable whether it was present at all. Therefore, the mutant and wildtype strain were tested in a chromogenic Limulus (LAL) assay, with meningococcal medium as a negative control. This assay depends on activation of the clotting enzyme

cascade in amoebocyte lysate prepared from the horseshoe crab and is capable of detecting picogram quantities of endotoxin. The results of the LAL assay on cell suspensions showed no significant endotoxin activity for H44/76[pHBK30] over meningococcal medium (0.3 and 1.7 EU/ml, respectively), in contrast to 21.7×10^4 EU/ml for the wildtype.

Taken together, these results demonstrate that the initial attempt to replace the wildtype *lpxA* gene with the hybrid construct resulted in the isolation of what was apparently an LPS-deficient mutant. This was further confirmed by gas-chromatography/mass-spectrometry (GC-MS) analysis of fatty acids present in OMC preparations, which showed that the lipid A-specific 3-OH C12 was present only in trace amounts in the mutant. As this fatty acid is added in the first step of lipid A biosynthesis, its absence demonstrates that the mutant is truly LPS-deficient and not just making some incomplete precursor molecule with no antibody binding or LAL assay activity.

Although H44/76[pHBK30] is fully viable, a reduced growth rate compared to the wildtype strain was apparent. When grown overnight on GC agar plates, the mutant strain produced much smaller colonies; in liquid medium the doubling time during exponential growth was approximately 50% higher than in wildtype strain H44/76.

The morphology of H44/76[pHBK30] and its parent strain was examined by electron microscopy of ultrathin sections. In contrast to the wildtype, cells of H44/76[pHBK30] were more heterogeneous in size and a significant fraction showed signs of lysis. However, the outer membrane could be clearly discerned in the LPS-deficient mutant (fig.5). In contrast to the somewhat "baggy" appearance in the wildtype, the outer membrane of the mutant showed a "tighter fit", possibly indicating a lowered rate of synthesis.

Example 2: Construction of an *lpxA* knockout mutant

An *lpxA* knockout mutant of *N.meningitidis* was constructed by inserting a kanamycin-resistance cassette into the BstEII site located at position 293 within the *lpxA* gene of plasmid pLA21 (a pUC18 derivative with a 2.1 kb *lpxD-fabZ-lpxA* insert). The resulting plasmid pLAK33 was digested with *XbaI/SacI* and transformed to strain H44/76 with selection for kanamycin-resistance. As expected, the resulting colonies showed the same growth properties as the H44/76[pHBK30] mutant, indicating the lack of LPS. This was confirmed by a whole cell ELISA in which the *lpxA* knockout mutant did not bind any of the LPS-specific mAbs. These results

demonstrated once more that blocking of the lipid A biosynthesis pathway in *N. meningitidis* strain H44/76 leads to viable LPS-deficient mutants.

Detailed description of the methods and strains used in the examples.

Where no specific details are provided standard technology has been applied. Where references are provided the content thereof is to be considered incorporated herein.

Bacterial strains and plasmids

The *E.coli* strains NM522 and INVαF' were grown on LB medium at 37°C. The *N.meningitidis* strain H44/76 and its derivatives were grown at 37°C on GC medium base (Difco) supplemented with IsoVitaleX (Becton Dickinson) in a humid atmosphere containing 5% CO_2, or in liquid medium as described (van der Ley et al., 1993). For selection of meningococcal transformants (van der Ley et al., 1996) kanamycin was used in a concentration of 75-100 µg/ml. With *E.coli*, antibiotics were used in the following concentrations: ampicillin, 100 µg/ml; kanamycin, 100 µg/ml. For cloning of PCR fragments, the TA cloning kit with the vector pCRII (Invitrogen) was used. Another vector used was pUC18.

Recombinant DNA techniques

Most recombinant DNA techniques were as described in Sambrook et al. (1989). Plasmid DNA was isolated using the pLASmix kit (Talent). The polymerase chain reaction (PCR) was performed on a Perkin Elmer GeneAmp PCR system 9600 with Taq polymerase. Sequence analysis was performed with an Applied Biosystems automatic sequencer on double-stranded plasmid DNA templates (isolated with Qiagen columns) and with a cycle sequencing protocol.

LPS analysis

Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed in 4% stacking and 16% separating gels as described by Lesse et al. (1990). Proteinase K-treated, boiled bacterial cells were used as samples. The gels were run for 17 h at a constant current of 20 mA, and silver stained by the method of Tsai and Frasch (1982). The chromogenic LAL assay for endotoxin activity was performed using the QCL-1000 kit from BioWhittaker Inc. (Walkersville, MD, USA) according to the instructions of the manufacturer. Overnight cultures were diluted in meningococcal medium to an OD at 620 nm of 0.1, and

serial dilutions of these stocks were used as samples in the LAL assay. For fatty acid analysis by GC-MS, OMC samples were acetylated for 3 h at 90°C in pyridine and acetic acid anhydride in order to completely dissolve the LPS. The samples were subsequently heated for 3 h at 65°C in tetrahydrofuran in the presence of $LiAlH_4$ to reduce the O-linked fatty acids to the free alcohols. These were derivatized to TMS-ethers for 1 h at 60°C with BSTFA + 1% TMCS in pyridine, and analyzed by GC-MS on an Autospec (Micromass, Manchester, UK) in the electron impact mode. The amount of 3-OH C12 in the samples was quantified using 2-OH C12 as internal standard.

Characterization of OMP composition

Binding of mAbs specific for class 1, 3 and 4 OMPs and for the oligosaccharide part of immunotype L3 LPS was tested in a whole-cell ELISA (van der Ley et al., 1995, 1996). Isolation of OMCs by sarkosyl extraction and their analysis by SDS-PAGE were done as described previously (van der Ley et al., 1993).

LEGENDS TO THE FIGURES

Figure 1. Construction of H44/76[pHBK30]. Two-step PCR mutagenesis leading to the hybrid *lpxA* gene, with *E.coli*-specific primers Epr1 (ACT-GACGCGTGTGATTGATAAATCCGC) seq. id. nr. 1 and Epr2 (GTAGGGCGGCACGTCCTGCGCCACACCGGA) seq. id. nr. 2 and *N.meningitidis*-specific primers Npr1 (TCCGGTGTGGCGCAGGACGTGCCGCCCTAC) seq. id. nr. 3 and Npr2 (CGGCCGCTCTAGAACTAGTGGATCA) seq. id. nr. 4.

Figure 2. Construction of H44/76[pHBK30]. Replacement of the chromosomal *lpxD-fabZ-lpxA* locus with the pHBK30 insert, carrying in addition to the *E.coli-N.meningitidis* hybrid *lpxA* gene a *kanR* selection marker instead of the 99 bp region between the MluI site in *fabZ* and the start codon of *lpxA* .

Figure 3. SDS-PAGE analysis of H44/76[pHBK30]. Silver-stained Tricine-SDS-PAGE LPS gel of proteinase K-treated whole-cell lysates of H44/76 (lanes 1 and 8) and six independent kanamycin-resistant transformants with pHBK30 (lanes 2-7).

Figure 4. SDS-PAGE of OMC proteins from H44/76[pHBK30] (lane 2) and H44/76 wildtype (lane 3); lane 1 contains a molecular weight marker of 94, 67, 43, 30, 20.1 and 14.4 kDa.

Figure 5. Electron micrograph of an H44/76[pHBK30] thin section, showing

the presence of the outer membrane in the absence of LPS.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(B) STREET: P.O. box 1
(C) CITY: Bilthoven
(D) STATE: Utrecht
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 3720 BA

(ii) TITLE OF INVENTION: Novel mutants of gramnegative mucosal bacteria and application thereof in vaccines.

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: E. coli

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACTGACGCGT GTGATTGATA AATCCGC 27

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: E. coli

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTAGGGCGGC ACGTCCTGCG CCACACCGGA 30

(4) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N. meningitidis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(5) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N. meningitidis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGGCCGCTCT AGAACTAGTG GATCA 25

ART 34 AMDT

CLAIMS

1. Gram-negative mutant of a mucosal bacterium, comprising a mutation such that it is viable, is capable of OMP formation and lacks endotoxic lipopolysaccharide (LPS), the mutant being free of LPS.

2. Gram-negative mutant according to claim 1 comprising a mutation such that it is free of Lipid A.

3. Gram-negative mutant according to claim 1 or 2, said bacterium being selected from the group comprising diplococci.

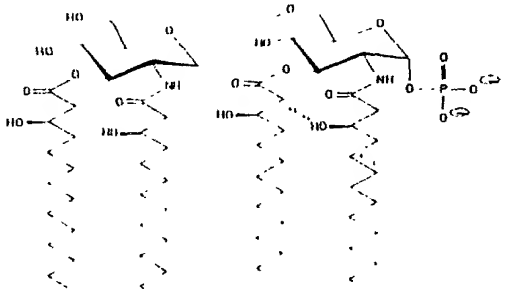
4. Gram-negative mutant according to any one of claims 1-3, said bacterium being selected from the group comprising gonococci, e.g. *N. gonorrhoeae*.

5. Gram-negative mutant according to any one of claims 1-3, said bacterium being selected from the group comprising meningococci e.g. *N. meningitidis*.

6. Gram-negative mutant according to claim 1 or 2, said bacterium being selected from the group comprising *Bordetella*, e.g. *Bordetella pertussis*.

7. Gram-negative mutant according to any on of the preceding claims, said mutant comprising a mutation in at least one gene associated with Lipid A biosynthesis.

8. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least one gene associated with the early stage of Lipid A biosynthesis, said early stage being prior to formation of the following structure



AMENDED SHEET

9. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least one gene selected from the group comprising *lpxA*, *lpxD* and *lpxB*.

10. Gram-negative mutant according to any one of the preceding claims, said mutant comprising a mutation in at least the gene *lpxA*.

11. Attenuated live vaccine against a gram-negative mucosal bacterium, said vaccine comprising a mutant according to any one of claims 1-10 as an active component and a pharmaceutically acceptable carrier.

12. Whole cell vaccine against a gram-negative mucosal bacterium, said vaccine comprising a mutant according to any one of claims 1-10 as an active component and a pharmaceutically acceptable carrier.

13. OMP vaccine against a gram-negative mucosal bacterium said vaccine comprising OMP derived from a mutant according to any one of claims 1-10 as an active component and a pharmaceutically acceptable carrier.

14. Vaccine according to any one f claims 11-13 further comprising an adjuvant.

15. Vaccine according to any one of claims 11-14 said vaccine being substantially free of endotoxic LPS, wherein substantially free is defined as LPS-free according to the Limulus assay.

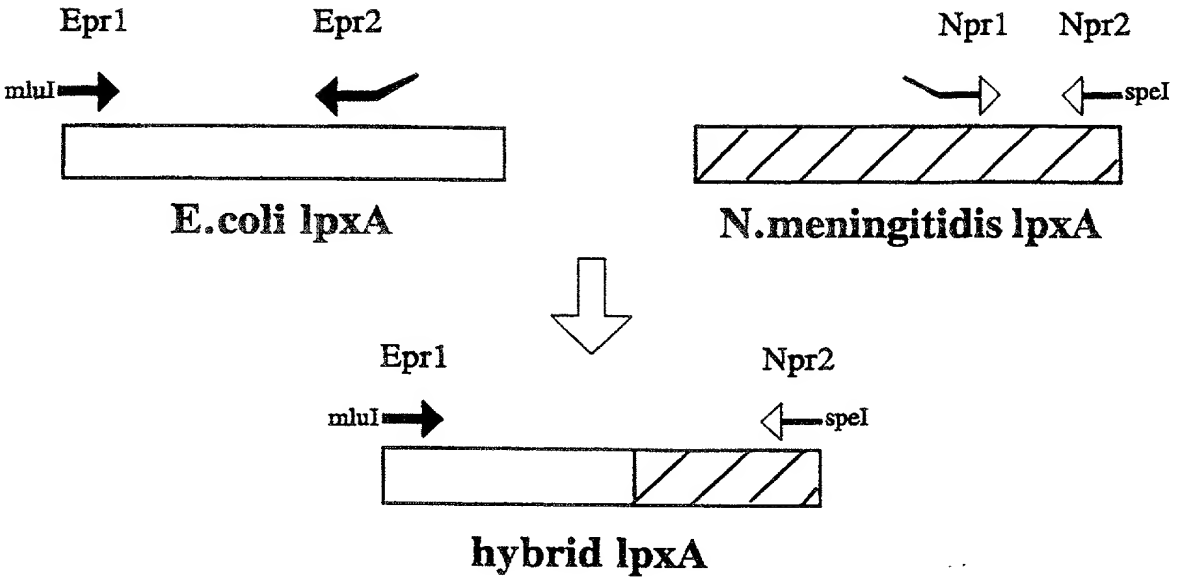
16. A method of producing LPS-free vaccine comprising application of a mutant according to any one of claims 1-10 and/or a part derived from said mutant as active component of a vaccine in a manner known per se for preparing vaccine formulations, said method being free of measures to remove LPS by purification.

17. A method of producing LPS-free OMP comprising culturing a mutant according to any one of claims 1-10 and deriving an OMP comprising fraction from said culture in a manner known per se for isolating protein from bacterial cuture, said method being free of

additional measures to remove LPS from said culture or OMP comprising fraction.

18. OMP which is free of LPS.

fig-1



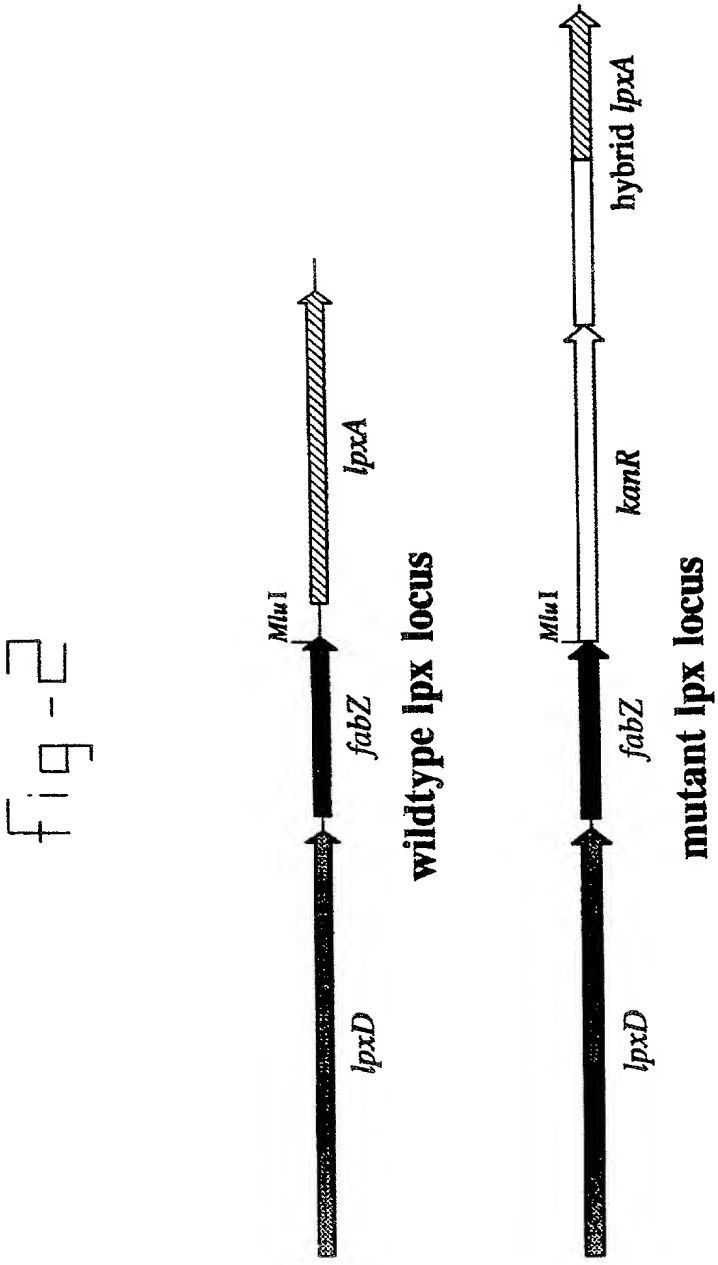

MluI
lpxA
fabZ
lpxD
wildtype lpx locus
MluI
hybrid lpxA
kanR
fabZ
lpxD
mutant lpx locus

Fig - 2

Figure 3

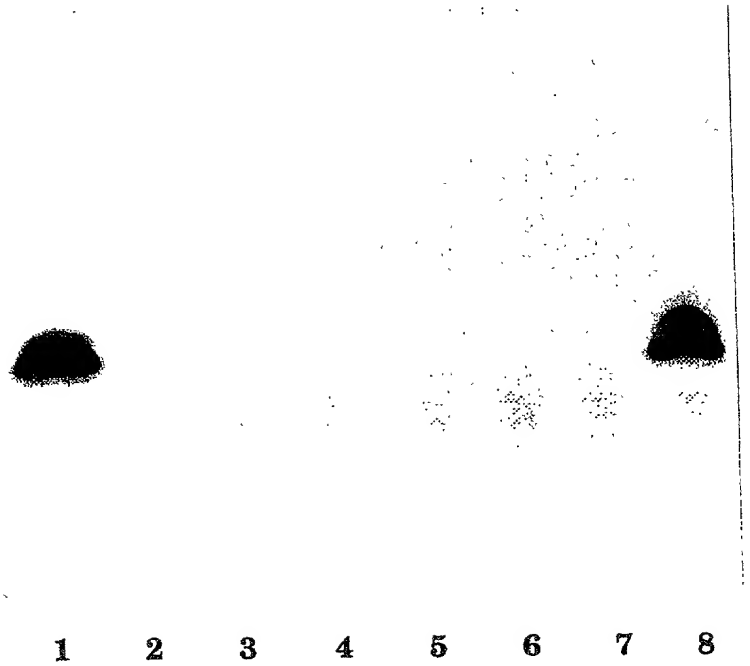


Figure 4

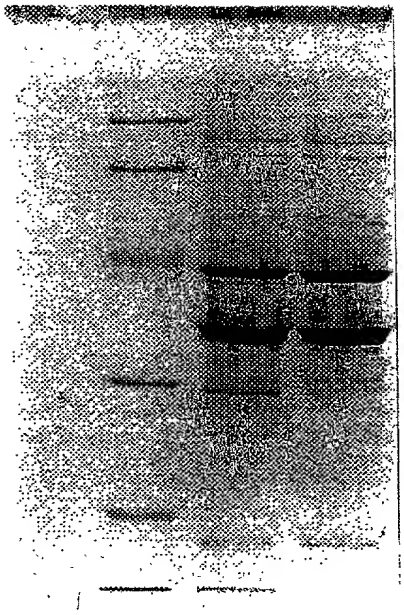
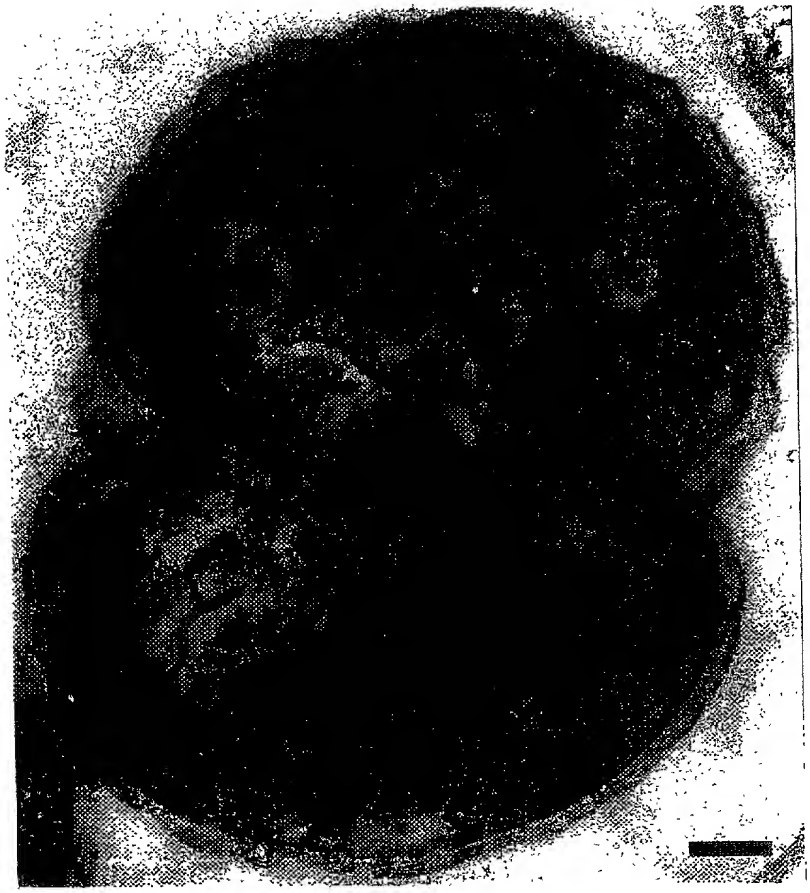


Figure 5



COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL DESIGN, NATIONAL STAGE OF PCT OR CIP APPLICATION)

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Novel mutants of gram negative mucosal bacteria and application thereof in vaccines

the specification of which: (complete (a), (b) or (c) for type of application)

REGULAR OR DESIGN APPLICATION

a. [ ] is attached hereto.

b. [ ] was filed on as Application
Serial No. and was amended on
(if applicable)

PCT FILED APPLICATION ENTERING NATIONAL STAGE

c. [X] was described and claimed in International application No.PCT/NL97/00474
filed on 21 August 1997
and as amended on (if any)

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, paragraph 1.56(a).

In compliance with this duty there is attached an information disclosure statement 37 CFR 1.97

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35. United States Code paragraph 119 of any foreign application (s) for patent of inventor's certificate listed below and have also identified below any foreign application for patent of inventor's certificate having a filing date before that of the application on which priority is claimed.

(complete (d) or (e))

d. [ ] no such applications have been filed
e. [ ] such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION

Country	Application Number	Date of filing (day, month, year)	Date of issue (day, month, year)	Priority claimed
–	–	–		–

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION

____

____

CONTINUATION-IN-PART

(Complete this part only if this is a continuation-in-part application)

I hereby declare claim the benefit under Title 35, United States code, paragraph 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claim of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, paragraph 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, paragraph 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

____

(Application Serial No.) (Filing date) (Status) (patented, pending, abandoned)

____

(Application Serial No.) (Filing date) (Status) (patented, pending, abandoned)

POWER OF ATTORNEY

6 – **As a named Inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Eric Jensen, Reg. No. 37,855, and Thomas W. PERKINS, Reg. No. 33,027 c/o YOUNG & THOMPSON, Second Floor, 745 South 23rd Street, Arlington, Virginia 22202.**

Address all telephone calls to Young & Thompson at 703/521-2297.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature

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Inventor's signature

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